Attachment and entry of *Candida famata* in monocytes and epithelial cells

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Running title: Morphology of the adhesion and entry of *C. famata* into cells

ABSTRACT

Candida albicans is considered the main pathogenic yeast responsible for a multitude of infective disorders. However, other yeasts, such as *Candida famata*, are being recognized as potential emerging pathogens that cause several types of infections in humans and animals. In this sense, we have investigated the adhesion and internalization of *Candida famata* into monocytes and epithelial cells. The interaction of the yeast with the cells is very rapid and takes place during the first 15 min of incubation. However, the affinity of the yeast for the cells varies, being highest for THP-1 (human monocytes), HeLa (human carcinoma), HaCaT and Pam-212 (human and mouse keratinocytes, respectively). Heat inactivation, or treatment with nystatin, significantly decreases yeast adhesion to cells. Immunofluorescence, as well as scanning and transmission electron microscopy (SEM and TEM, respectively) analysis, reveals that cell lines are able to internalize *C. famata*. At 48 h after infection, most of the yeasts located inside cells appear degraded, but some yeasts, recovered from lysed cells, were still viable. Adhesion and internalization of *C. famata* into HeLa cells were found to be lower than those of *C. albicans*, and *C. glabrata,* but higher than those of *S. cerevisiae*. In addition, infection with *C. famata* results in actin microfilaments rearrangement. This paper presents novel data in the interaction of this pathogenic yeast with mammalian cells.

Keywords: *Candida famata*; Yeast attachment; Cellular infection; Actin alteration.

INTRODUCTION

Fungal infections caused by *Candida* species are increasing particularly in immunocompromised individuals. Infection in such patients often results in a very high mortality rate, more than 60% in some cases, due mainly to systemic dissemination. *C. albicans* is the most common fungal opportunistic pathogen isolated from blood cultures and blood-associated infections. However, infections by other yeast species are becoming increasingly frequent, particularly in the nosocomial setting. These emerging yeasts, which include at least 17 *Candida* species, are being recognized as human pathogens and include: *C. parapsilosis, C. tropicalis, C. krusei, C. glabrata, C. lusitaniae, C. guilliermondii, C. famata, C. kefyr, C. rugosa, C. stellatoidea* and*, C. norvegensis* (Carlile et al., 2001; Fries and Casadevall, 2001; Krcméry and Barnes, 2002; Bustamante, 2005; Ruhnke, 2006; Saalwachter at al., 2006). *C. famata*, in particular, has been poorly studied although it has been isolated from foods and from human and animal tissues (Andrighetto et al., 2000; Gardini et al., 2001; Krcméry and Kunová, 2000; Nishikawa et al., 1996; Rao et al., 1991). However, in most cases, it has been considered as a contaminant and only in a few cases it has been directly related to human infections. At present, it is thought to be the pathogen responsible for approximately 0.2 to 2% of human candidiasis, including ocular diseases (Rao et al., 1991; Carrasco et al., 2005), peritonitis (Quindos et al., 1994), mediastinal candidiasis (Ahmed et al., 2005), candidiasis in neonates (Heljic et al., 2005) and general fungemia (Carrega et al., 2000; Krcméry and Kunová, 2000). This yeast can also infect bone (Wong et al., 1982) and central nervous tissues (Prinsloo et al., 2003) and more recently it has been implicated in other human infections (Abia-Bassey and Utsalo, 2006; Carrasco et al., 2005).

Some of the most extensively investigated virulence factors of *Candida* include its ability to attach to host tissues and to penetrate these tissues (Gow et al., 2002; Rooney et al., 2002; Filler, 2006; Argimon et al., 2007). Attachment of *Candida* to host cells is the initial stage in the infectious process, enabling the microorganisms to survive inside the host and eventually colonize the host tissues during the development of candidiasis. Although several components of the fungal cell wall such as chitin, β-glucans and lipids may participate in the adhesion process, specific surface proteins and mannoproteins (adhesins) are the most important mediators in such interaction (Filler, 2006). A number of adhesins that promote the virulence in *C. albicans* and also in *C. glabrata* have been

described (Filler, 2006; Kaur et al., 2005). All these proteins interact with extracellular matrix components (fibronectin, laminin, fibrinogen or collagen) and a variety of host receptors of phagocytic and non-phagocytic host cells, including Toll-like receptors (2 and 4) and N-cadherin adhesion protein (Alberti-Segui et al., 2004; Filler and Sheppard, 2006; Filler 2006; Newman et al., 2005; Phan et al., 2005). The patterns of response to infection are most probably due to different recognition of these receptors rather than dimorphism (yeast/hyphae transition), which is considered as an important virulence factor in *Candida* species (Filler, 2006; Filler and Sheppard, 2006; Lorentz et al., 2004).

After adhesion of the yeast to the cell membrane receptor, the next step in the infection process is its internalization into the host cell. Once inside the cell, the majority of the yeasts are eventually degraded, although some may survive within the phagosome (Filler, 2006; Filler and Sheppard, 2006; Lorentz et al., 2004; Romani et al., 2002). Internalization and yeast survival can be observed in *in vitro* systems in which macrophages or other non-phagocytic cells are infected by *C. albicans.* Once inside the cells, the yeast form of *C. albicans* differentiates into the filamentous hyphal form and may induce cell host lysis (Filler and Sheppard, 2006; Lorenz and Fink, 2002; Lorenz et al., 2004; Romani et al., 2002).

Given that *C. famata* can be considered as an emerging pathogen associated with a range of infectious conditions (eye, intravenous catheter infections, fungemia and peritonitis) in humans, particularly in immunocompromised patients, this work was designed to study the ability of this yeast to attach to monocytes and epithelial cells (keratinocytes and carcinoma cells) *in vitro*, as well as to determine the morphological changes related to the entry of the yeast into the cells. These cells lines have been chosen as study models since all of them are relevant to Candida infection: monocytes being phagocytic and defence cells and epithelial cells as they constitute the first barrier in the infection step. The results presented in this work indicate that *C. famata* can successfully adhere to, and enter, these mammalian cell lines, providing novel data in the interaction of this yeast with cells *in vitro.*

MATERIALS AND METHODS

Yeasts and growth conditions

Candida famata, Candida albicans, Candida glabrata and S. cerevisiae were maintained as frozen stocks until culturing at 30 ºC on YPD (1% of yeast extract, 2% peptone and 2% glucose) with 20% agar (YPD-agar). Aliquots from agar cultures adjusted to a concentration of 0.5×10^3 to 2×10^3 colony-forming units (CFU)/ml, were routinely grown overnight to stationary phase in YPD medium at 30 ºC with continuous shaking. After incubating for 24 h, the turbidity was estimated by visual reading at $\lambda = 600$ nm in a Shimadzu UV-1601 spectrophotometer. Prior to cell infection, yeast cultures were then diluted in fresh medium and grown to logarithmic phase for 3 h. *C. famata* was isolated in our laboratory from a patient with acute zonal occult outer retinopathy (AZOOR) (Carrasco et al., 2005); *C. albicans* (SC5314); *C. glabrata* (MCYC 2267) and *S. cerevisiae* were obtained from the Spanish Collection of Culture (University of Valencia, Spain)

Morphological studies

Aliquots of *C. famata* cultures taken during the logarithmic phase were centrifuged for 10 min at 1,200 rpm and the yeast was fixed in cold 95% ethanol (-20 ºC) for 5 min. After fixing, yeast suspensions were again centrifuged and finally resuspended in distilled water. Yeast smears were stained either with toluidine blue (0.05% for 1 min, Sigma, TB) or with Hoescht-33258 (5 μg/ml for 3 min, H-33258) to determine the general or nuclear morphology, respectively. Some smears were stained with calcofluor white (1 μg/ml for 3 min, Sigma). After washing, smears were air dried and mounted in DePex (Serva).

Cell lines

The following cell lines were used as models for analyzing adhesion and internalization of *C. famata*: Pam-212: (murine keratinocytes), HaCaT (human keratinocytes) and THP-1 (human monocytic cells). Pam-212 and HeLa and HaCaT cells were grown in Dulbecco's modified Eagle medium (DMEM) and THP-1 cells in RPMI 1640 (medium with Glutamax-1). All media contained 10% (v/v) fetal calf serum (FCS), 50 units/ml penicillin, 50 μg/ml streptomycin, and 1% (v/v) 0.2 M L-glutamine (all from Gibco). Cell cultures were carried out at 37 ºC under a humidified 5% CO_2 atmosphere in F25 flasks or on 22-mm square coverslips placed in 3 cm dishes.

Adhesion assays

Adhesion assays were carried out according to Cormack et al., (1999) with some modifications. Cells grown to a 70-80% confluence, either adhered to coverslips or in suspension (THP-1), were exposed to *C. famata*. Yeasts, grown to logarithmic phase, were first centrifuged for 10 min at 1,200 rpm, resuspended in DMEM, MEM or RPMI and then added to the cell cultures (at about 10 x 10^6 yeasts per 3 cm plate, corresponding to a multiplicity of infection of approximately 10 to 1). After incubation at 37 ºC in 5% CO_2 for periods ranging from 15 min to 3 h, non-adherent yeasts were removed by washing first with PBS (4-5 times) followed with 1% EDTA in PBS (4 times). Then, cells were either incubated further in a complete medium or fixed for 6 min in cold methanol (-20 ºC), air dried and stained with TB. In the case of THP-1 cells, samples of the infected culture in suspension were taken, dropped onto coverslips, air dried, fixed with methanol and processed as attached cells. Attachment of *C. famata* to the different cell lines was quantified as the percentage of cells with at least one yeast adhered to the cell membrane. The adhesion ability of *C. famata* was then compared to that of *C. albicans, C. glabrata* or *S. cerevisiae* in the HeLa human cells. The experiments were repeated at least three times and about 1000 cells were counted for each experimental condition.

Immunofluorescence

Indirect immunofluorescence was performed according to Carrasco et al. (2005) and to Espada et al. (2005) to determine: (1) the characteristics of the yeast wall under different experimental conditions and (2) the adherence and the uptake of the yeast by the mammalian cultured cells. Immunofluorescence was also performed to compare adhesion and internalization of *C. famata* with *C. albicans* or *S. cereviseae*. Cells subjected to infection with yeasts were washed extensively with PBS, fixed with 3.5% paraformaldehyde for 15 min and permeabilized with 0.5% triton X-100 (Sigma) in PBS. Cells were then exposed to rabbit polyclonal antibodies against *C. famata, C. albicans, C. glabrata or Saccharomyces cerevisiae* (1:1000 in PBS-BSA 2%) for 1 h at 37 ºC, washed with PBS and incubated with the secondary antibody (FITC labeled goat anti-rabbit IgG) (Sigma) for 1 h at 37 ºC. Some cells infected with *C. famata* were also subjected to F-actin detection by incubating the coverslips with TRITC-labeled phalloidin (Sigma). Finally, the coverslips were washed again in PBS, counterstained with 2.5 μg/ml H-33258 for 3 min,

dehydrated and mounted in DePex. Microscopy observations and photography were performed with an Olympus photomicroscope IMT-2, equipped with an HBO 100 W mercury lamp and the corresponding filter sets for fluorescence microscopy: ultraviolet (UV, 365 nm, excitation filter UG-1), blue (450-490 nm, excitation filter BP 490) and green (545 nm, excitation filter BP 545). Effects on the actin cytoskeleton were also studied with confocal laser microscopy using a Leica confocal microscope equipped with a 25 mW krypton-argon laser and a 10 mW helium-neon laser (488, 543 nm).

Assessment of internalization of yeasts

Cells grown on coverslips were exposed to the yeast for 3 h at 37 ºC DMEM in the absence of serum. Cells were washed 4-5 times with PBS and postincubated in complete medium at 37 ºC for different periods (up to 48 h). Cells were then fixed and submitted to one of the following procedures: processing for immunofluorescence detection, staining with TB or H-33258, or preparation for scanning electron microscopy (SEM) or transmission electron microscopy (TEM). Internalization of *C. famata* into the different cell lines was quantified for cells subjected to immunofluorescence as the percentage of cells with positive fluorescence within the cytoplasm. Internalization assessment of *C. albicans, C. glabrata* or *S. ceresisiae* was also performed in human Hela cells. In the case of cells infected with *C. albicans*, internalization was only studied at 3h after infection, since yeast/hyphae transition was detected at longer times, thereafter being difficult to determine hyphae internalization.

In order to determine whether yeasts were internalized or simply bound to the surface of the cells, corresponding controls using indirect immunofluorescence were performed. To this end, infected cells were fixed and, without permeabilization, immediately subjected to the antiserum; thereafter, cells were permeabilized and exposed to the secondary antibody. The experiments were repeated at least three times and about 1000 cells were counted for each experimental condition.

Inactivation of C. famata

In order to determine whether yeast adhesion and/or internalization depended on cell viability, *C. famata* was inactivated by heating or by treatment with the antifungal nystatin (NYST). Inactivation by heating was performed prior to cell infection by warming

the yeast suspension for 15 min at 90 ºC in YPD. The yeast suspension was then centrifuged (10 min at 1,200 rpm), resuspended in RPMI and added to the cultured cells. In addition, NYTS was administered at a final concentration of 5 μg/ml during cell infection in the culture medium (Pfaller et al., 2003). After infection, cultured cells were washed 5 times with PBS to remove non-adhered yeast, and fixed or incubated for a further 24 h in DMEM containing NYST. Cells were then washed again in PBS and stained with TB or processed for immunofluorescence studies. Effects of heating or NYST treatment on yeast viability were studied by seeding aliquots of treated yeasts on agar plates and determining the presence of CFUs.

SEM and TEM studies

For SEM, cells interacting with *C. famata* were washed with PBS and fixed in 3% glutaraldehyde (Taab Laboratories) in PBS for 1 h. After washing with PBS, cells were postfixed with 1% osmium tetroxide (Taab Laboratories) in PBS, treated with graded series of ethanol, air dried, coated with gold (15 nm) using a Sputter Coater SC502 and then examined with a Phillips SEM at 20 kV.

For TEM, cells were fixed for 2 h with a solution containing 4% PFA-2% glutaraldehyde in sodium cacodylate (0.1 M, pH 6.8), washed with buffer, scraped, centrifuged (10 min at 1000 rpm), dehydrated in graded series of ethanol and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate.

For immunodetection in TEM, cells were fixed in 4% PFA-0.1% glutaraldehyde in phosphate buffer (0.1 M pH 7.4), washed in the buffer, scraped, centrifuged and embedded in 10% gelatin in PBS. Gelatin pieces containing the cells were gradually infiltrated with saccharose (from 0.1 to 2 M) at 4 ºC and frozen by immersing the pieces in liquid nitrogen. Thereafter, pieces were cryosubstituted in methanol containing 0.5% uranyl acetate for 50 h at -85 ºC, washed in methanol and embedded in Lowicryl HM20 (Polysciences, Inc. Warrington, PA.). Polymerization was performed under UV light for 1-2 days at -45 ºC and for a further 2-3 days at room temperature. Ultrathin sections were exposed to rabbit anti-*C. famata* antiserum (1:4000), washed and incubated with the secondary antibody, goat anti-rabbit IgG conjugated to 10-nm gold particles (1:40, British Biocell Int.). All these ultrathin sections were examined in a Jeol JEM1010 TEM at 80 kV. Images were obtained using a Bioscan digital camera (Gata, Pleasanton, CA, USA).

Determination of cell viability

The viability of mammalian cells infected with *C. famata* was determined by the thiazolyl blue (MTT) (Sigma) assay 48 h after infection (Galaz et al., 2005). MTT in DMEM-1% FCS was added to cell cultures at a final concentration of 50 μg/ml for 3 h and reduced formazan was then dissolved in DMSO and absorption measured at 540 nm. The effects of treatments were expressed as the percentage of cell lethality induced by *C. famata* in comparison with uninfected cells. The viability of internalized *C. famata* was assessed by recovering the yeasts through lysis of the mammalian cell monolayers in 0.1% Triton X-100, growing them for 24 h on YPD plates and determining the number of CFUs formed. All the viability experiments were repeated at least three times.

Statistical analysis

The unpaired *t*-test was used to compare differences between experimental groups. Differences were considered statistically significant when $P<0.05$. Data shown represent the mean ± SD of three independent experiments performed in triplicate.

RESULTS

C. famata morphology: Adhesion and Entry into mammalian cells.

C. famata was initially stained using different assays. Figure 1 shows the morphology of *C. famata* in the exponential growth phase when unicellular forms and/or dividing cells were the most abundant forms. Complexes of multicellular forms comprising a small number of connected (chain) yeasts were also present. The general morphology of the yeast can be observed after staining with TB (Fig. 1A). The yeast wall determined by indirect immunofluorescence using a rabbit antiserum was well defined (Fig. 1B). The bud scars are visualized by calcofluor staining (Fig. 1C) and the ultrastructural characteristics of unicellular or multicellular forms of *C. famata* were observed using TEM (Fig. 1D). All these morphologies were present in the yeast suspensions employed to infect mammalian cells. The ability of these *C. famata* forms to attach to the different cell lines investigated (epithelial cells: HeLa, HaCaT and Pam-212; and monocytic cells: THP-1) was observed after both TB and H-33258 staining (Fig. 2). Adhesion to cells occurred soon after addition

of *C. famata* and, 15 min later, most mammalian cells presented a variable number of yeasts attached to their surfaces. Adhesion seemed to be lowest in the murine Pam-212 cell line and highest in the monocytes THP-1. In the infected cultures of this THP-1 cell line, a population of adherent cells with macrophage morphology appeared after infection. These macrophages presented yeast attached to the plasma membrane or internalized within them (Fig. 2, THP-1). At 3 h after infection, most cell lines exhibited a marked morphological alteration. This alteration was manifest through increased vacuolization of the cytoplasm, which was particularly noticeable when TB staining was employed (Fig. 2, Pam-212). In addition, some nuclear morphological changes were observed under the fluorescence microscope with Hoescht-33251 staining (Fig. 2, HaCaT). Nevertheless, the majority of the cells had recovered the control morphology at 48 h after infection.

Adhesion, determined by TB staining at different times (15 min and 3 h) after cell infection and quantified by counting the number of cells with attached yeasts, is shown in Figure 3 and in Table 1. The adhesion values estimated soon after infection (15 min) were maintained at 3 h in all mammalian cell types. Adhesion at 3 h was lowest in the epithelial murine line Pam-212, which formed tight cellular colonies, followed by the epithelial lines HeLa and HaCaT with colonies organized in a less compact fashion; monocytic THP-1 cells presented the highest number of attached yeasts (53 ± 8.2%). Although there were no significant differences between 15 min and 3 h in this assay, the infection time of 3 h was selected for the analysis of short-term interaction between the cell lines and yeasts because adhesion rates should have stabilized.

The internalization of *C. famata* was also analyzed by TB staining and by immunofluorescence of permeabilized cells (Fig. 3, Table 1). The altered morphology of cells induced by the attachment and entry of the yeasts became manifest through vacuolization of the cytoplasm, as clearly shown after TB staining (Fig. 3a). Green fluorescent yeasts could be observed inside cells soon after infection (3 h) (Fig. 3b). In addition, the number of yeasts in the cell cytoplasm was, in general, maintained during the first hours after infection. These results indicate that *C. famata* was being internalized into both phagocytic (THP-1) and non-phagocytic cells (Table 1). The percentage of cells with green fluorescent yeasts within the cytoplasm at 3 h after infection was quite similar to the adhesion values: 2.7 ± 0.5% for Pam-212, 5.6 ± 0.5% for HaCaT, 10 ± 2% for HeLa, and 56 ± 6% for THP-1 cells. These values progressively decreased in all the cells; fluorescing

points gradually disappeared and only a few cells exhibited positive reaction indicating that most of the yeast were degraded. It should be noted that fluorescing points corresponded to yeasts in all cases, since no fluorescence was detected in the parallel controls of internalization (indirect immunofluorescence on infected fixed cells, subjected to the primary antiserum, then permeabilized and exposed to the secondary antibody). The viability of internalized *C. famata,* assessed by recovering the yeasts through lysis of the mammalian cells and growing them on YPD plates, indicated that 48 h after infection, most of the yeasts located inside the cells appeared degraded, but some were still viable. In addition, *C. famata* infection affected cell viability, though cell death rates were low (around 10%) in all the cell types (evaluated by the MTT test 48 h after infection) (Table 1).

Adhesion and internalization of *C. famata* was compared in the HeLa cell system, representative of non-phagocytic adherent cells, with those of the pathogen yeasts *C. albicans* and *C. glabrata* and with the non pathogen yeast *S. cerevisiae* (Fig. 4). Adhesion rates were very similar for *C. famata, C. albicans* and *C. glabrata,* but lower for *S. cerevisiae* at early times (3 h) after infection. However, adhesion rates significantly increased with time in the case of *C. albicans* and *C. glabrata,* whereas adhesion of *C. famata* and *S. cerevisiae* remained relatively constant with time. Internalization of *C. glabrata* was higher than that of *C. famata*, both being higher than that of *S. cerevisiae*, which was very limited (less than 3% of the infected cells showed positive reaction in immunofluorescence). It should be noted that internalization of *C. albicans* could only be evaluated at 3 h after infection since, thereafter, the appearance of yeast/hyphae transition made it difficult to determine its internalization. The morphology of HeLa cells infected with *S. cerevisie* (A), *C. glabrata* (B) or *C. albicans* (C-C´´) after TB staining is also shown in Figure 4.

Infection by C. famata induces alterations in actin microfilaments in mammalian cells

In view of the important role played by actin microfilaments in phagocytosis processes, we proceeded to analyze the changes produced in the distribution of this element of the cytoskeleton during the first hours after *C. famata* infection (attachment and internalization) in adherent HeLa (Fig. 5A-A´´´) and Pam-212 (Fig. 5B-B´´, C-C´) cells and THP-1, representive of phagocytic and non-adherent cells. After infection, cells were

fixed, submitted to *C. famata* immunofluorescence detection, stained for F-actin with phalloidin-rhodamine (phalloidin-TRICT) and analyzed by fluorescence microscopy (Fig. 5A-A´´´, B-B´´) or by confocal microscopy (Fig. 5C-C´). As shown in Figure 5 (A, B), both epithelial cells exhibited a fairly well developed network of F-actin cytoskeleton organized as stress fibers and located at the cell cortex. Yeast attachment affected actin microfilament organization. The microfilaments appeared partially disorganized, as reflected by an absence of stress fibers and diffuse fluorescence during the first hours after infection, especially at the adhesion site (Fig. 5A´-A´´, B¨). These F-actin alterations were maintained in the majority of the cells, either with or without apparent fluorescent points within the cytoplasm at 24 h after infection (Fig. 5A´´´, B´´). However, at 48 h after infection, cells recovered the control morphology as regards to F-actin organization (results not shown). Confocal microscopy revealed that *C. famata* adhered to or was internalized into Pam-212 cells (Fig. 5C, C´). Internalization of the FITC-labeled yeasts into cells is demonstrated by the intercalation of the *C. famata* between F-actin fibers. Z-sections through the cells revealed the intercalation of the yeast between F-actin fibers, thus indicating the internal location in the cells. In addition, F-actin appears also altered in the THP-1 cells (Fig. 6). Whereas control THP-1 cells exhibited a quite well developed subcortical F-actin (Fig. 6A), cells with internalized *C. famata* showed deep alterations in actin microfilaments (Fig. 6B). In addition, nuclei of infected cells showed picnotic chromatin (Fig. 6B´) in contrast with those of controls (Fig. 6A´), as it can be observed under the fluorescence microscope after H-33258 staining.

Effect of different treatments on C. famata adhesion to cells

The specificity of the interaction of *C. famata* with mammalian cells was analyzed in two selected cell lines: HeLa, (adherent cells) and THP-1, (phagocytic and non-adherent cells). In this experiments, *C. famata* was submitted to heat treatment or treated with the antifungal compound nystatin. Treatments were carried out before or during the infection procedure. Both treatments reduced the ability of *C. famata* to bind HeLa or THP-1 cells (Fig. 7). In HeLa cells, adhesion decreases to 35 ± 1.5% after heating, 28 ± 1.6% with NYST (Fig. 7). On the other hand, 24 h, heat treatment reduced yeast internalization (37 ± 2.1%), whereas NYST did not alter the entry of *C. famata* (Fig. 7). The results for THP-1 cells were similar, with a decrease of adhesion rates of 79 ± 0.3% with heating and 27 ±

0.8% with NYST. Also, as in the case of HeLa cells, the treatments employed reduced the internalization of the yeast by 89 ± 0.1% with heating and 88 ± 0,2% with NYST. These results indicate that both adhesion and internalization of *C. famata* within HeLa and THP-1 seem to be active infection processes.

Entry of C. famata analyzed by electron microscopy

To better determine the morphological processes of yeast attachment and internalization, SEM and TEM studies were performed (Figs. 8 and 9). SEM revealed adhesion and entry of *C. famata* into HeLa cells in detail (Fig. 8); these results being in agreement with those obtained after TB or immunofluorescence microscopy. The microphotographs show that *C. famata* was attached to the surface of HeLa cells both as yeast and as cell chains. Interphasic or mitotic cells contain several yeast cells inside them. After adhesion, the cells develop thin or broad cytoplasmic projections such as filopodia or lamellipodia (Fig 9A-A´, B-B´, C-C´). These projections envelop the yeasts as the infection progresses; once engulfed, the yeast inside the cytoplasm produces a well-defined deformation of the cell surface (Fig. 8D-D´).

The adhesion and internalization of *C. famata* cells were also characterized by TEM in HeLa and in THP-1 cells (Fig. 9). The yeast wall presents numerous protrusions ("fimbriae" like) (Krautgartner et al., 2003), which interact with the cell plasma membrane of HeLa cells during the adhesion process, in a well-organized fashion at the contact sites (Fig. 9A-A´´). At the attachment sites, the plasma membrane of cells appeared thicker and denser than the membrane not interacting with the yeast (Fig. 9A´´). Yeast internalization can be observed in different cells containing yeasts in their cytoplasm (Fig. 9C-E). The structure of these engulfed yeasts was variable; some reflected morphologies similar to those of controls, whereas other internalized yeasts exhibited clear signs of phagolysosome digestion, especially in THP-1 cells (Fig. 9E´´). After infection there are clear alterations in the morphology of the infected cells over time. These alterations, however, do not seem to significantly affect cell viability (see: Table 1). Conversely, the morphology of most yeasts did not show significant changes during the first hours after infection, remaining very similar to controls. Nevertheless, as the culture progressed, many yeasts developed important morphological alterations, mostly in the cytoplasm, which underwent intense

plasmatic membrane retraction, cytoplasmic vacuolization and wall deformation (Fig. 9B,E).

DISCUSSION

Systemic infections due to *Candida* species other than *C. albicans* are increasingly responsible for morbidity and mortality in humans, particularly in hospitalized and immunodepressed patients (Bustamante, 2005; Krcméry and Barnes, 2002; Ruhnke, 2006; Saalwachter, 2006). In addition, some of these species, including *C. famata*, seem to be less susceptible to traditional systemic antifungal agents (Bustamante, 2005; Krcméry & Barnes, 2002; Ruhnke, 2006; Saalwachter at al., 2006). Therefore, it is of interest to investigate the interaction of these species with mammalian cells. The results obtained in this study indicate that *C. famata* can bind and be internalized not only into monocytes (THP-1) but also epithelial carcinoma cells (HeLa) and keratynocytes (HaCaT and Pam-212). The mechanisms of cell infection by *Candida* species are not well known, and most of the available data correspond to studies performed in *in vitro* and particularly for *C. albicans*, the most infectious in humans. According to these studies, the virulence of *Candida* species may be affected by several factors such as dimorphic transition, adhesion to a biological substrate, antigenic variability and production and secretion of photolytic enzymes, as well as the defence mechanisms of the host during the infection processes (Carlile et al., 2001; Krcméry and Barnes, 2002; Gow et al., 2002; Filler and Sheppard, 2006).

In the conditions of the present study, *C. famata* is able to adhere to the cell surface of mammalian cells studied and become internalized. The infective capacity not necessarily depends on the morphology of the fungi. Although the majority of dimorphic micellar fungi, such as *C. albicans*, is to be found in the form of yeast cells, their conversion to hyphae seems to be an important but not essential element for pathogenesis and tissue invasion. Moreover, both fungal forms, yeast and hyphae, are important for the initiation and progression of infection (Filler and Sheppard, 2006; Kurzai, 2005; Rooney and Klein, 2002; Spellberg et al., 2005). Yeast cells have their own ways of promoting pathogenesis: (1) they can adapt better to the circulation system or to the host tissues, (2) they can avoid the immune system by residing within macrophages and (3) it seems likely

that the different yeast morphologies are responsible for its dissemination in blood (Rooney and Klein, 2002). In our case, *C. famata* does not form mycelia, is mainly found in unicellular forms and it can invade cultured cells.

The first stages of the process of cell infection are very similar to phagocytosis. However, whereas the ingestion of a pathogenic organism in phagocytosis results in its elimination by enzymatic degradation (Boyle and Finlay, 2003; Filler, 2006; Filler and Sheppard, 2006; Gardini et al., 2001; Ghoneum et al., 2003), in the infection process, the ingestion of the yeast does not result in its destruction. In both cases, the first step is the binding of the pathogen to the cell surface, followed by the subsequent formation of cell projections around it and, finally, the pathogen is internalized. Our present findings indicate that interaction of *C. famata* with human and murine cells occurs quickly, within the first hour of infection and that this yeast does not exhibit the same ability to bind to the different cell types. The cell line with the highest yeast binding capacity are the monocytes THP-1, followed by the cells of epithelial origin: HeLa and HaCaT, and Pam-212. *C. albicans,* both in the yeast and hyphal phases, binds to a broad range of host tissues, including components of the extracellular matrix, platelets, epithelial (such as HeLa cells), and endothelial cells, macrophages, leucocytes (Filler and Sheppard, 2006; Filler et al., 2006; Romani et al., 2002; Sundstrom, 2002). Also *C. glabrata* is able to bind to several cell types (Kaur et al., 2005; Sundstrom, 2002). In addition, our results show that, for the same cell type (HeLa cells), the adhesion values are higher for the pathogen yeasts *C. albicans* and *C. glabrata,* than for *C. famata*; all of the three being higher than those of *S. cerevisiae*. These variations in the adhesion values may be related to a different number of adhesion and/or receptor molecules for the different yeasts (being highest for *C albicans* and *C. glabrata* and lowest for *S. cerevisiae)* which, in turn, could be related to their different infective ability.

On the other hand, the fact that intact *C. famata* showed a higher adhesion rate than after inactivation by heating or by NYST treatment indicates that the yeast could have an active role in the adhesion and internalization processes. These results agree well with the results reported for *C. albicans* in endothelial cell cultures, where higher adhesion was detected for live yeasts (Filler et al., 1995; Filler, 2006). These differences in adhesion may indicate that the receptors involved in the adhesion and internalization processes of the yeast are not exactly the same. Thus, SEM analysis indicates that both filopodia and

lamellipodia are formed in the adhesion process of *C. famata* although the different roles of the two structures in the phagocytosis are not clear. In dendritic cells, filopodia and lamellipodia have been described as participating in the adhesion and ingestion of yeast and hyphal forms of *C. albicans*, respectively (Romani et al., 2002; Rooney and Klein, 2002; Zamze et al., 2002). In the case of *C. famata*, the results obtained after TEM, SEM and fluorescence studies indicate that, under our experimental conditions, most of the yeasts that have invaded the cells are finally digested. However, some of them resist the digestion being responsible for the mortality rate ($<10 \pm 3\%$) observed after infection in all the cell lines examined.

On the other hand, alterations in the actin microfilaments of the host HeLa and Pam-212 epithelial cells during adhesion and internalization of *C. famata* have been observed. Actin rearrangements also occur in non-infected cells, suggesting that some factor or factors that alter the actin cytoskeleton may be secreted by the yeast. In fact, several studies have reported that other fungal pathogens, particularly *C. albicans,* induce visible alterations in the cytoskeleton of non-phagocytic eukaryotic cells to promote their internalization, intracellular motility and survival (Tsarfaty et al. 2000; Chen et al., 2003; Ibrahim-Granet et al., 2003; Larsson, 2006; Sandovsky-Losica and Segal, 2006). However, the results obtained in this study on the infection of mammalian cells with *C. famata* suggest that actin cytoskeleton modifications are a consequence of such an interaction; the mechanisms of this process and its implications in the infection process still need further investigation. In conclusion, the present results indicate that *C. famata* can infect HeLa (non-phagocytic) and THP-1 (phagocytic) cells, as well as other mammalian cell types, being able to induce alterations in microfilaments and cell death. Nevertheless, further studies should be performed to determine whether the uptake is a host-mediated event, a pathogen-mediated event, or is due to both.

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LEGEND OF FIGURES

Figure 1.- Morphology of *C. famata* grown in YPD during the exponential phase. A: TB staining; B: indirect immunofluorescence; C**:** Calcofluor staining; D: TEM. *C. famata* grows in the unicellular or multicellular forms (chains). Scale bar: 2 μm.

Figure 2.- A: Attachment of *C. famata* to mammalian cells: HeLa, HaCaT, Pam-212, and THP-1. A: control cells stained with TB; B and C: cells infected for 3 h with *C. famata* and stained with TB (B) or with H-33258 (C), respectively. H-33258 staining was observed under UV excitation light. *C. famata* attaches to cells either in interphase or during division (white arrow) and induces extensive cytoplasmic vacuolization (black arrows). Control THP-1 shows cells in suspension, whereas adherent macrophages are present in infected cultures (Infection, TB). Scale bar: 10 μm.

Figure 3.- A: Comparison of the adhesion rates of *C. famata* to the different cell lines at 15 min and 3 h after infection. After staining with TB, the adhesion rate was calculated by counting the cells with at least one yeast attached to their plasma membranes. No significant differences were found between 15 min or 3 h of incubation, with the monocytic cells presenting the highest estimated adhesion rate and the keratinocytes the lowest. B: Internalization kinetics of *C. famata* into mammalian cells as a function of the elapsed time after infection (3-48 h). Internalization of *C. famata* into cells was determined by TB staining or immunofluorescence of the yeast. The experiments were repeated at least 3 times. * $P< 0.05$ compared to adhesion or internalization rates of infection. Yeasts within the cell cytoplasm are observed added to cell surface (arrow) or inside cell vacuoles after TB staining (a, arrow head) and as fluorescing points due to immunofluorescence detection (b) 24 h after infection in the examples shown (HeLa or THP-1 cell, respectively). Scale bar: 5 μm.

Figure 4.- Adhesion and internalization of *C. famata* compared with *C. albicans*, *C. glabrata* and *S. cerevisiae* in the HeLa cell system. As shown, both adhesion and internalization rates of *C. famata* were lower than those of *C. albicans* and *C. glabrata*, but higher than those of *S. cerevisiae*. TB staining of HeLa cells infected with *S. cerevisie* (A),

C. glabrata (B) or *C. albicans* (C) 3 h after infection. *C. albicans* yeast/hyphae transition observed 6 and 24 h after infection is shown in C′-C′′. Scale bar: 10 μm.

Figure 5.- Effects of the adhesion and internalization of *C. famata* on actin microfilaments of HeLa (A-A′′′) or Pam-212 (B-B′′, C-C′) cells. *C. famata* was determined by immunofluorescence (FITC-labeled) and actin microfilaments by phalloidin-TRITC staining. Actin microfilaments (fluorescing in red) of HeLa and Pam-212 cells appear well organized at subcortical region and stress fibers in both cell types (A,B). Yeasts fluorescing in green are adhered to the cell surface (A′,B′) (head arrows) and inside (A′′-A′′′,B′′) (arrows) cells. 3 h after infection, actin fibers are partially disorganized in both cell types, especially at the adhesion sites (A′,B′). Actin fibers disorganization is also patent 6 h (A′′) and 24 h (A′′′,B′′) after infection and diffuse fluorescence can be observed in the cell cytoplasm. Internalization of *C. famata* is corroborated by confocal microscopy in Pam-212 cells (C,C′); Z-sections perpendicular to the focal image plane confirm the presence green fluorescence due to yeast between the red fluorescence of actin microfilaments. Scale bar: 10 μm.

Figure 6.- Actin rearrangements of THP-1 cells observed 3 after infection with *C. famata*. Control cells show a well developed subcortical actin, whereas in infected cells is altered. Nuclei of infected cells stained with H-33258 are deformed and polarized. Scale bar: 10 μm.

Figure 7.- Variations in the percentage of yeast adhesion and entry into HeLa or THP-1 cells induced by different treatments and evaluated at 3 h (adhesion) or at 24 h (internalization) after infection. C: cells infected with untreated yeast; HT: cells exposed to heated yeast prior to infection; NYST, BFA: cells infected with the yeast in the presence of nystatin or brefeldin A, respectively. The adhesion and internalization values obtained were referenced to controls (C).

Figure 8.- SEM analysis of the adhesion and internalization of *C. famata* in HeLa cells. Interphasic (A,A′) or mitotic (B,B′) cells bind several yeast cells at the same time. The development by the cells of cytoplasmic projections such as lamellipodia (A′,C′) or

filopodia (B´,C) are observed. Engulfed yeasts induced deformations of the mammalian cell surface (D,D´). Scale bar: 2 μm.

Figure 9.- Characterization of the infection processes of HeLa (A-D) and THP-1 cells (E-F) by TEM. Interaction of the yeast projections (fimbriae like) with the plasma cell membrane shown at different magnifications (A,A´,A´´). The cell membrane appears thicker at the binding sites (A´,A´´) (white arrow). Sequential phases of yeast internalization into HeLa cells (B,C,D) or THP-1 cells (E-E´´) can be seen. Internalized yeast inside HeLa cells reacting positively to *C. famata* antibody are observed in C,C´. Engulfed and partially digested yeasts can be seen inside phagolysosomes of THP-1 cells (E´´). Some THP-1 cells with extensive vacuolization can also be seen (F). Scale bar: 3 μm.

	Pam-212	HaCaT	HeLa	THP-1
Adhesion (% ± S.D.)	8 ± 0.26	15 ± 0.4	15 ± 0.3	53 ± 8.2
Entry (% ± S.D.)	2.7 ± 0.5	5.6 ± 0.5	10 ± 2	56 ± 6
Cell survival (% ± S.D.)	91,5 ± 6	93,5 ± 4	90,5 ± 3	88,5 ± 4

Table 1.- Attachment and entry of *C. famata* in epithelial cells: Pam-212 (murine), HaCat, HeLa (human) or monocytes (human, THP-1). Evaluation of attachment and entry was performed at 3 h or 24 h, respectively, after infection by TB staining and/or immunofluorescence detection. Mammalian cell survival was evaluated 24 h after *C. famata* infection by the MTT test. Data correspond to mean values ± standard deviation (S.D.) from three different experiments.